Chapter III

Paracrine Factors of Human Amniotic Fluid-derived Mesenchymal Stem Cells Show Strong Anti-fibrotic Properties by Inhibiting Myofibroblast Differentiation and Collagen Synthesis

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Abstract

Objective: Myofibroblasts are causally involved in the hallmark of fibrosis, i.e. the excessive deposition of a collagen-rich extracellular matrix (ECM). So far, there are no pharmacological treatments that combat successfully fibrosis, making this pathology a major global disease burden. In preclinical models, mesenchymal stem cells attenuate fibrosis, but how these stem cells are involved remains unclear. In this study, we assessed the effect of paracrine factors of fetal and adult human stem cells on primary dermal myofibroblasts.

Methods: TGFβ1-activated human adult dermal (myo)fibroblasts (two donors: age 27 and 73 years) were treated with conditioned medium collected from amniotic fluid-derived stem cells (cmAFSCs) as well as from adipose tissue-derived stem cells (cmADSCs). The effects of conditioned medium on the following fibrogenic events were measured: formation of myofibroblasts, synthesis of ECM as well as cell proliferation.

Results: Cell proliferation was enhanced. The main pro-fibrotic effects of TGFβ1, namely the induction of myofibroblast formation (αSMA) and collagen type I protein synthesis, were blocked to baseline levels with cmAFSCs. Similar data were obtained for the ECM-proteins tenascin C, fibronectin, and collagen type III. Furthermore, pre-existing myofibroblasts could be reversed into fibroblasts. Synthesis of lysyl hydroxylase 2, a collagen-modifying enzyme, was highly up-regulated despite the absence of the major fibrillar collagens, and it is speculated that this enzyme has also another function. The use of bFGF-neutralizing antibodies revealed that the suppression of αSMA stress fibers by cmAFSCs can be partially attributed to bFGF. Despite the fact that cmADSCs was also able to reverse pre-existing myofibroblasts into fibroblasts, its anti-fibrotic properties was less compared with cmAFSCs. Major discrepancies between mRNA levels and protein levels were observed, especially for collagen type I.

Conclusions: This study describing the high anti-fibrotic potential of conditioned medium from human fetal stem cells on adult fibroblasts cultured under pro-fibrotic conditions.

Key words

Fibrosis, Fibroblasts, Myofibroblasts, TGFβ1, Mesenchymal Stem Cells, and Extracellular Matrix.

Introduction

Fibrosis is characterized by an unwanted accumulation of extracellular matrix (ECM) molecules, in particular fibrillar collagen type I, leading to scar formation and subsequently to tissue dysfunction and/or organ failure. Despite the fatal outcome of fibrosis in diseases such as end-stage liver disease, kidney disease, idiopathic pulmonary fibrosis and heart failure, so far no pharmacological therapies are in place to successfully attenuate or even halt fibrosis [1]. Therefore, other strategies are under investigation to reduce fibrosis, such as the use of stem cells, or the paracrine factors derived thereof.

Adult mesenchymal stem cells invariably attenuate fibrosis in a number of experimental mouse and rat models [2-6]. Fetal mesenchymal stem cells also attenuate, like their adult counterparts, fibrosis in a variety of organs (e.g. liver, kidney, lung, and myocardium) in preclinical models [7-18]. Most likely the effects are paracrine-mediated, as the application of conditioned medium (CM) from stem cells has similar effects [8, 19, 20], and as the number of stem cells engrafted in the tissue is too low to have a direct effect on the tissue properties themselves [8, 11, 15, 17]. Since the hallmark of fibrosis is the deposition of large quantities of ECM molecules (e.g. collagen) by activated fibroblasts (i.e. myofibroblasts) [21, 22], it follows that in all these cases less collagen/myofibroblasts must be present, which is indeed reported. However, a variety of processes are involved in the repair of damaged tissues, such as angiogenesis, inflammation, and cell recruitment, processes that can all contribute to the outcome of fibrosis (i.e. the number and activation state of the *in situ* fibroblasts). Consequently, in these *in vivo* studies the direct effects of paracrine signaling of stem cells on fibroblasts cannot be investigated due to the complexity of the micro-environment in which the fibroblasts are embedded.

One approach to overcome this problem is to investigate the effects of stem cell-derived CM on fibroblasts and myofibroblasts cultures *in vitro*. Such studies have indeed been carried out, both with CM from fetal [23-30] and adult [5, 6, 31-38] stem cells. Unfortunately, conflicting data have been reported, for example with respect to collagen, where an up-regulation [23, 24, 28-34, 37, 38] as well as a down-regulation [5, 6, 25, 26, 35, 36] is reported. Apart from that, anti-fibrotic effects of CM from fetal or adult stem cells on (myo)fibroblasts have never been compared, although such a comparison is potentially interesting, given the fact that in the fetal environment scarring normally does not occur [39, 40]. Here we investigate the effect of CM of amniotic fluid-derived human mesenchymal stem cells (cmAFSCs) and CM of human adult mesenchymal stem cells derived from the stromal-vascular fraction of subcutaneous adipose tissue (cmADSCs) on primary adult human dermal fibroblasts derived from a donor aged 27 and 73 years (HDF-27 and HDF-73, respectively). Our main finding is that cmAFSCs strongly suppress the differentiation of fibroblasts into myofibroblasts, as well as the production of ECM molecules (collagen type I and III, fibronectin and tenascin C), and that cmADFCs has less pronounced effects.

Materials and methods

Ethical approval

The research was conducted in accordance with the ethical rules for human experimentation as stated in the 1975 Declaration of Helsinki and was approved by the Medical Ethical Committee of the University Medical Center Groningen, The Netherlands. Adipose tissue was obtained with informed consent from healthy donors undergoing elective abdominal liposuction surgery at Bergman Clinics, Heerenveen, The Netherlands. Confluent "back-up" human amniocentesis cultures were received, after informed consent of the patients, from the clinical cytogenetics laboratory of the University Medical Center Groningen. Only cells from healthy fetuses (with a normal karyotype) were used. Human dermal fibroblasts were commercially available from ATCC and Cell Applications as described in the cell culture section.

Cell culture

AFSCs from four healthy fetuses were obtained from the clinical cytogenetics laboratory of the University Medical Center Groningen as described previously [41]. AFSCs were cultured and expanded in Dulbecco's Modified Eagle Medium (DMEM) (Lonza, Switzerland) supplemented with 1% L-glutamine (Lonza), 1% penicillin/streptomycin (Gibco Life Technologies Ltd., UK) and 20% fetal bovine serum (FBS) (Thermo Scientific, USA). After reaching confluency, cells were subsequently cultured in Eagle's Minimal Essential Medium (EMEM) (Lonza) supplemented with 1% L-glutamine, 1% penicillin/streptomycin and 20% FBS (Thermo Scientific, USA). FACS analysis revealed that the cell populations were negative (< 4% positive cells) for the hematopoietic markers CD34 and CD45, positive for the mesenchymal markers CD44 (> 98%) and CD90 (> 65%) and the embryonic stem cell markers SSEA4 (> 70%) and Oct4 (> 94%) [41]. ADSCs were kindly provided by Dr. Guido Krenning (University Medical Center Groningen). The ADSCs were isolated from adipose tissue from three donors as described previously [42] and cultured under the same conditions as described for AFSCs.

HDF-27 was obtained from the skin of the forearm of a Caucasian male (age 27 years) and HDF-73 was obtained from the facial skin of a Caucasian female (age 73 years). HDF-27 (CCD-1093Sk (= ATCC CRL-211), ATCC, USA) and HDF-73 (106-05a, Cell Applications, San Diego, USA) were cultured in EMEM supplemented with 1% L-glutamine, 1% penicillin/streptomycin and 10% FBS. Passage 5 to 12 of HDF-27 and passage 3 to 6 of HDF-73 were seeded with a density of 15,000 cells/cm^2 in a Costar 12-well plate (for quantitative real time polymerase chain reaction) or a 48-well plate (Corning Inc., USA) (for immunofluorescence staining). Fibroblasts were washed with phosphate buffered saline (PBS) after 72 h, starved overnight, and subsequently cultured for the indicated time period in (1) EMEM supplemented with 1% L-glutamine, 1% penicillin/streptomycin and 0.5% FBS (= control), (2) idem, but with the addition of recombinant human TGFβ1 (100-21, Peprotech, UK) at a concentration of 5 ng/ml, (3) in CM of AFSCs with or without spiking of 5 ng/ml TGFβ1, and (4) in CM of ADSCs with or without spiking of 5 ng/ml TGFβ1. In all

experimental conditions, L-ascorbic acid 2-phosphate magnesium salt (Sigma A-8960, USA) was added at a concentration of 0.17 mM. This is a stable derivative of L-ascorbic acid; the latter is highly unstable in aerobic conditions, neutral pH and in solution. All cell culture protocols were carried out at 37°C in a humidified 5% CO_2 environment.

To decipher whether the bFGF in cmAFSC is involved in the observed suppression of αSMA and collagen type I synthesis by cmAFSC as described below, we pre-incubated each cmAFSC sample with a human bFGF neutralizing antibody (Sigma F-6162, USA) for 30 minutes at a concentration of 2.5 µg/ml. HDF-73 fibroblasts were subsequently stimulated with TGFβ1 in the presence of cmAFSCs with or without anti-bFGF for 48 h, followed by and qRT-PCR and immunocytochemistry analysis.

Preparation of conditioned medium (CM)

Passages 3 to 5 of AFSCs and ADSCs were seeded at a concentration of 15,000 cells/cm^2 in a T162 flask (Corning Inc., USA) for 72 h to grow them ~70% confluence. The attached cells were washed twice with PBS and incubated for 96 h with EMEM containing 1% L-glutamine, 1% penicillin/streptomycin and 0.5% FBS. The medium was filtered using a 0.2 µm syringe filter and centrifuged at 1250 rpm for 5 min. The prepared CM was then used to perform the experiments.

RNA isolation, cDNA synthesis and qRT-PCR

HDF-27 and HDF-73 were treated with/without TGFβ1 and/or CM of AFSCs and ADSCs for 48 h. Total RNA was isolated using the Favorgen RNA extraction kit (Favorgen Biotech, Taiwan) according to the manufacturer's protocol. The concentration and quality of RNA was checked with UV spectrophotometry (NanoDrop Technologies, Wilmington, NC). For the synthesis of cDNA, total RNA was reverse transcribed with the First Strand cDNA synthesis kit (Fermentas, Lithuania) according to the manufacturer's protocol. Gene expression analysis was performed by means of qRT-PCR in a 10 µl reaction mixture containing 10 ng cDNA, SYBR Green Master Mix (Roche, USA), 6 µM forward and 6 µM reverse primer (for primer sequences see Table 1). qRT-PCR was performed in triplicate for each condition in a 384-well plate at 95 °C for 15 sec and 60 °C for 1 min for 40 cycles using the ViiA 7 Real-Time PCR System (Applied Biosystems, USA). Data was analysed with the ViiA 7 Real-Time PCR System Software v1.1 (Applied Biosystems, USA).

Table 1: List of primer sequences used for qRT-PCR.

Gene	Forward sequence	Reverse sequence
ACTA2	CTGTTCCAGCCATCCTTCAT	TCATGATGCTGTTGTAGGTGGT
COL1A1	GCCTCAAGGTATTGCTGGAC	ACCTTGTTTGCCAGGTTCAC
COL3A1	CTGGACCCCAGGGTCTTC	CATCTGATCCAGGGTTTCCA
TNC1	CCGGACCAAAACCATCAGT	GGGATTAATGTCGGAAATGGT
FN1	CTGGCCGAAAATACATTGTAAA	CCACAGTCGGGTCAGGAG
PLOD2	ATGGAAATGGACCCACCAA	TGCAGCCATTATCCTGTGTC
YWHAZ	GATCCCCAATGCTTCACAAG	TGCTTGTTGTGACTGATCGAC

Immunofluorescence staining

HDF-27 and HDF-73 were stimulated with or without TGFβ1 and/or CM of AFSCs and ADSCs for 48 h. After treatment, cells were washed with PBS and fixed with methanol/acetone (1:1) for 5 min at – 20 ºC. Subsequently, cells were washed and incubated with primary antibodies (Table 2) diluted in PBS containing 2% bovine serum albumin (BSA) (K1106, Sanquin, Netherlands) for 1 h at RT. After washing with PBS, cells were incubated for 30 min at RT with biotinylated secondary antibodies (Table 3) diluted in PBS containing 2% BSA for 30 min at RT. The cells were washed again and incubated with streptavidine-CY5 (Invitrogen, USA) (1:100) in PBS containing 1% BSA and DAPI (1:10,000) for 30 min. After washing with PBS, cell culture wells were mounted with citifluor (Agar Scientific, UK) and the staining pattern was visualized by fluorescence imaging microscopy (TissueFAXS, TissueGnostics GmbH, Austria), and analyzed as described previously [43].

Table 2: List of primary antibodies used for immunofluorescence analysis.

Antigen (protein)	Antibody (Dilution)	Source (Cat. No, Company)
α-SMA	Mouse monoclonal IgG2a (1:100)	M0851, Dako, Denmark
Collagen I	Mouse monoclonal IgG1 (1:300)	ab90395, Abcam, UK
Collagen III	Mouse monoclonal IgG1 (1:200)	ab6310, Abcam, UK
Fibronectin	Rabbit polyclonal IgG (1:400)	ab6584, Abcam, UK
Tenascin C	Mouse monoclonal IgG1 (1:100)	ab6393, Abcam, UK
LH2	Mouse polyclonal IgG (1:100)	SAB1400213, Sigma, USA

Table 3: List of Secondary antibodies used for immunofluorescence analysis.

Antigen (protein)	Biotinylated Secondary Antibody (Dilution-1:100)	Source (Cat. No, Company)
α-SMA	Goat-anti-mouse IgG2a	1080-08, SouthernBiotech, USA
Collagen I, Collagen III, and Tenascin C	Goat-anti-mouse IgG1	1071-08, SouthernBiotech, USA
Fibronectin	Goat anti-rabbit IgG	E0432, Dako, Denmark.
LH2	Goat-anti-mouse IgG	1030-08, SouthernBiotech, USA

Statistics

All mRNA data were normalized against the reference gene tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ). All mRNA and immunofluorescence data are presented as mean ± SEM for three (ADSCs) or four (AFSCs) donors. Results were analysed with either one-way analysis of variance (ANOVA) followed by Tukey's post-test or two tailed unpaired *t*-test analysis using Graph-Pad Prism Version 5 (GraphPad Software Inc., USA). $P < 0.05$ was considered to be statistically significant. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. The sign # represents a statistically significant difference compared to the untreated control.

Results

Characterization of HDF-27 and HDF-73

The fibroblasts of the two donors differ significantly from each other with respect to mRNA levels of certain genes, as well as with regard to the synthesis of certain proteins. mRNA levels of ACTA2 and PLOD2 were significantly higher in HDF-73 compared to HDF-27 (Figure 1A). mRNA levels of COL3A1 were significantly lower in HDF-73, whereas mRNA levels of COL1A1, FN1 and TNC1 did not show significant differences between both donors (Figure 1A). An increased synthesis of αSMA and collagen type I in HDF-73 was seen with immunohistochemistry, indicating the presence of myofibroblasts (Figure 1B).

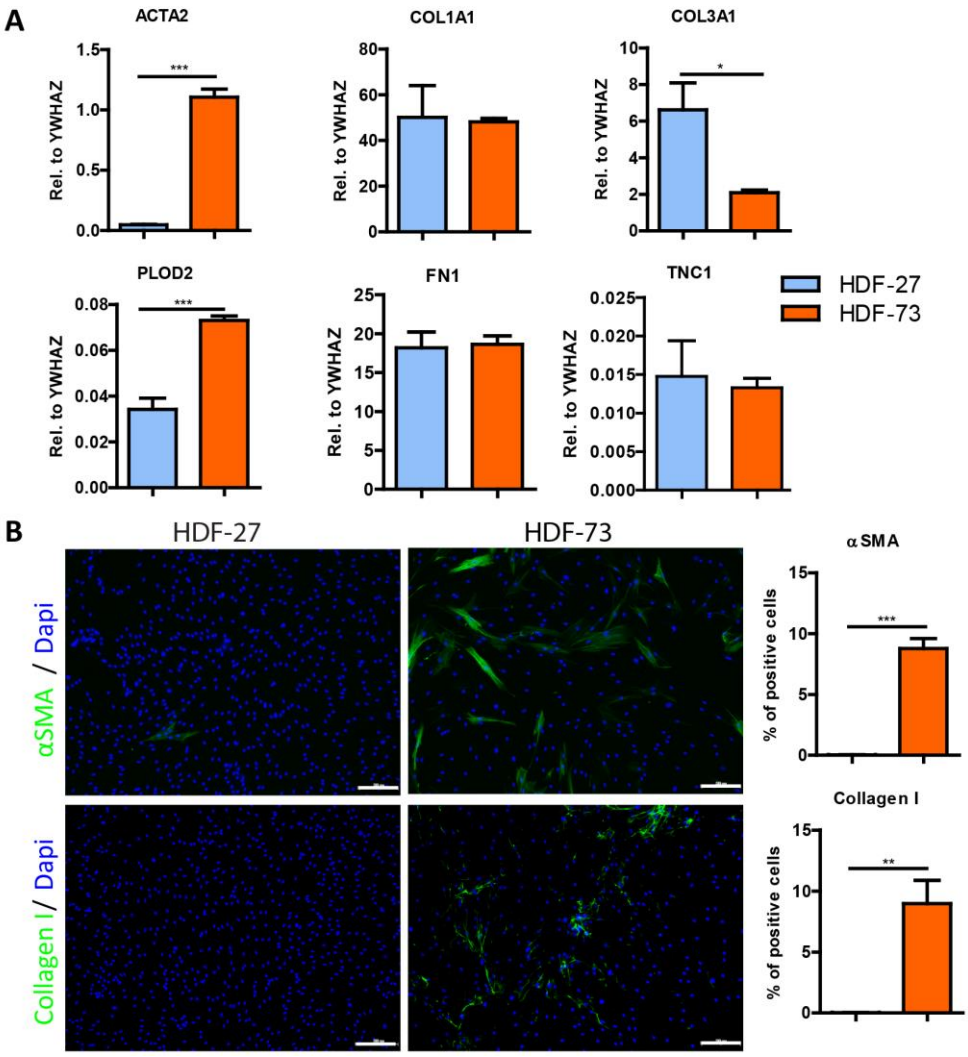


Figure 1. Basal expression of fibrosis-related molecules in HDF-27 and HDF-73. Fibroblasts were cultured for 48 h to measure gene and protein expression. (A) mRNA levels of ACTA2, COL1A1, COL3A1, PLOD2, FN1, and TNC1 were measured with qRT-PCR and expressed relative to the reference gene YWHAZ. (B) Representative immunofluorescence stainings and quantification of the % of cells positive for α-smooth muscle actin (upper panel) and collagen type I (lower panel) are shown for HDF-27 and HDF-73. White scale bar represents 200 µm.

Effect of cmAFSCs on myofibroblast differentiation and ECM-related molecules

We stimulated human dermal fibroblasts (HDF-27 and HDF-73) with TGFβ1 to induce the formation of myofibroblasts, i.e. cells that are involved in the deposition of excessive amounts of ECM. This resulted in a major increase in the mRNA levels of ACTA2 (Figure 2A), the gene that encodes for the protein αSMA, being a marker for myofibroblasts. Indeed, a major increase was seen in the number of αSMA-positive cells (Figure 2B). Incubation with TGFβ1 in the presence of cmAFSCs resulted in a suppression of ACTA2 mRNA levels and αSMA-positive cells back to baseline levels (Figure 2A-B), thus completely neutralizing the activation effect of TGFβ1. The same was seen with collagen type I: increased COL1A1 mRNA levels and increased collagen protein synthesis after TGFβ1 stimulation, which were suppressed to baseline levels with cmAFSCs (Figure 3A-B). Similar data were observed for collagen type III in HDF-27 (Figure 4A-B), as well as for tenascin C both in HDF-27 and HDF-73 (Figure 5A-B). COL3A1 mRNA levels in HDF-73 were not up-regulated after TGFβ1 stimulation, and collagen type III synthesis was not significantly reduced when co-incubated with cmAFSCs (Figure 4A-B). Fibronectin mRNA levels were up-regulated after TGFβ1 stimulation both in HDF-27 and HDF-73, but remained the same (HDF-27) or were even enhanced (HDF-73) in the presence of cmAFSCs (Figure 6A). Fibronectin protein synthesis was also up-regulated after TGFβ1 stimulation, and remained the same (HDF-27), or was, in sharp contrast to mRNA, reduced to baseline levels (HDF-73) in the presence of cmAFSCs (Figure 6B). PLOD2 mRNA levels were up-regulated after TGFβ1 stimulation, and were even more enhanced in the presence of cmAFSCs (Figure 7A). The same was observed for lysyl hydroxylase 2, the protein product of PLOD2 (Figure 7B).

Effect of cmADSCs on myofibroblast differentiation and ECM-related molecules

The same experiment was carried out for CM derived from ADSCs, and compared with AFSCs. We observed major differences. First, mRNA levels of ACTA2 were down-regulated only in HDF-27, but not to baseline levels (Figure 2A). The amount of αSMA-positive cells was not down-regulated, but even up-regulated in the case of HDF-27 (Figure 2B). mRNA levels of COL1A1 were down-regulated, but not to baseline levels, and only a relatively minor effect was seen on collagen synthesis (40% reduction for HDF-73, no reduction for HDF-27) (Figure 3A-B). mRNA levels of COL3A1 were not down-regulated, whereas suppression is seen in collagen type III production both in HDF-27 and HDF-73 (Figure 4A-B). Tenascin C mRNA levels did not change in HDF-27, and were increased in HDF-73, whereas no changes were seen for protein levels (Figure 5A-B). Fibronectin mRNA levels were not down-regulated, as was the case for protein production (Figure 6A-B). Finally, PLOD2 mRNA and protein levels were not increased (Figure 7A-B).

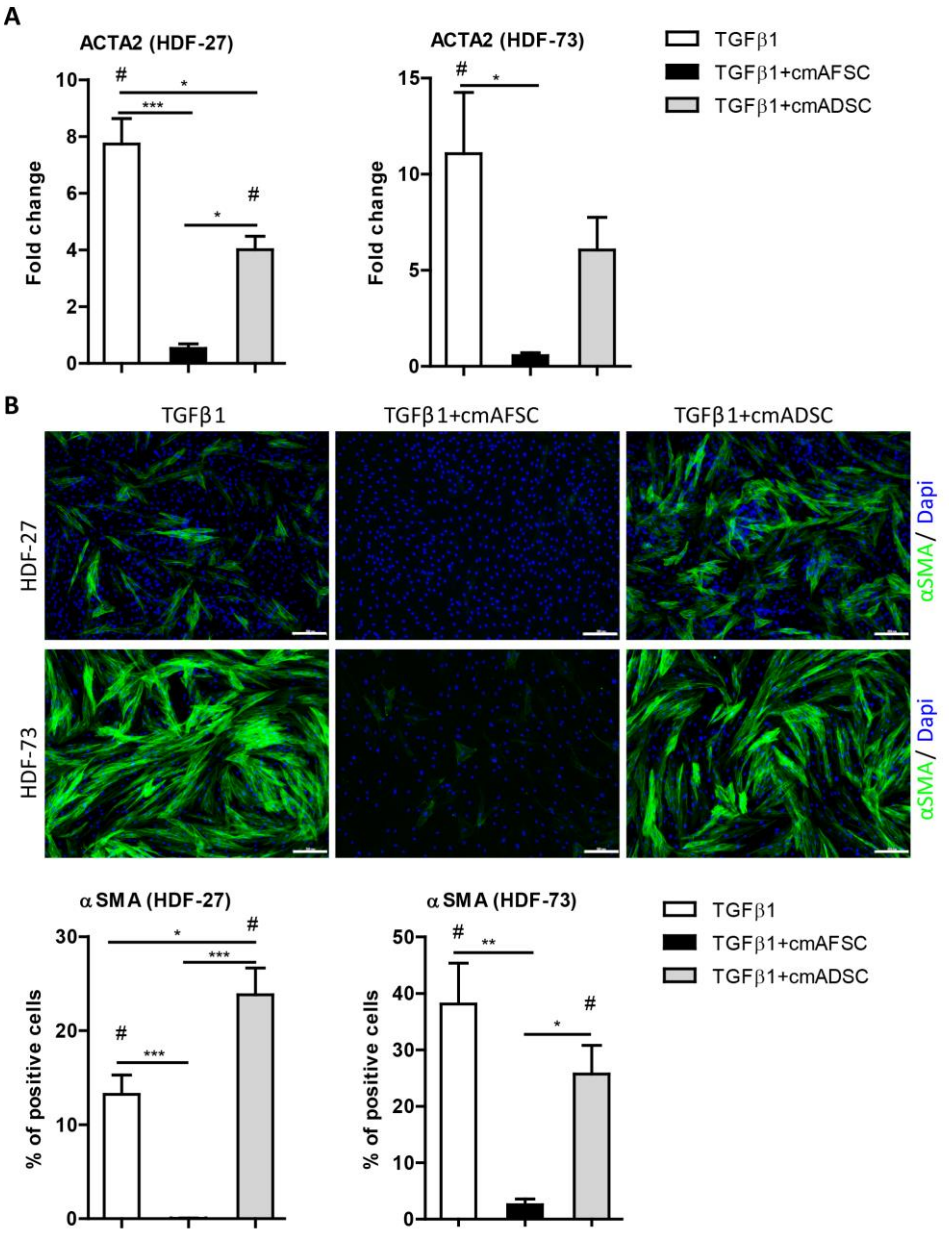


Figure 2. Effects of cmAFSCs and cmADSCs on TGFβ1-induced αSMA synthesis in HDF-27 and HDF-73. Fibroblasts were treated with or without TGFβ1 and in combination with either CM of AFSCs or ADSCs for 48 h. (A) mRNA levels of ACTA2 as measured with qRT-PCR and expressed as fold-change compared to untreated control (cells cultured in plain medium without TGFβ1). (B) Representative immunofluorescence stainings and quantification of the % of cells positive for αSMA in HDF-27 (upper panel) and HDF-73 (lower panel). White scale bar represents 200 µm. The sign # represents statistically significance towards the untreated control.

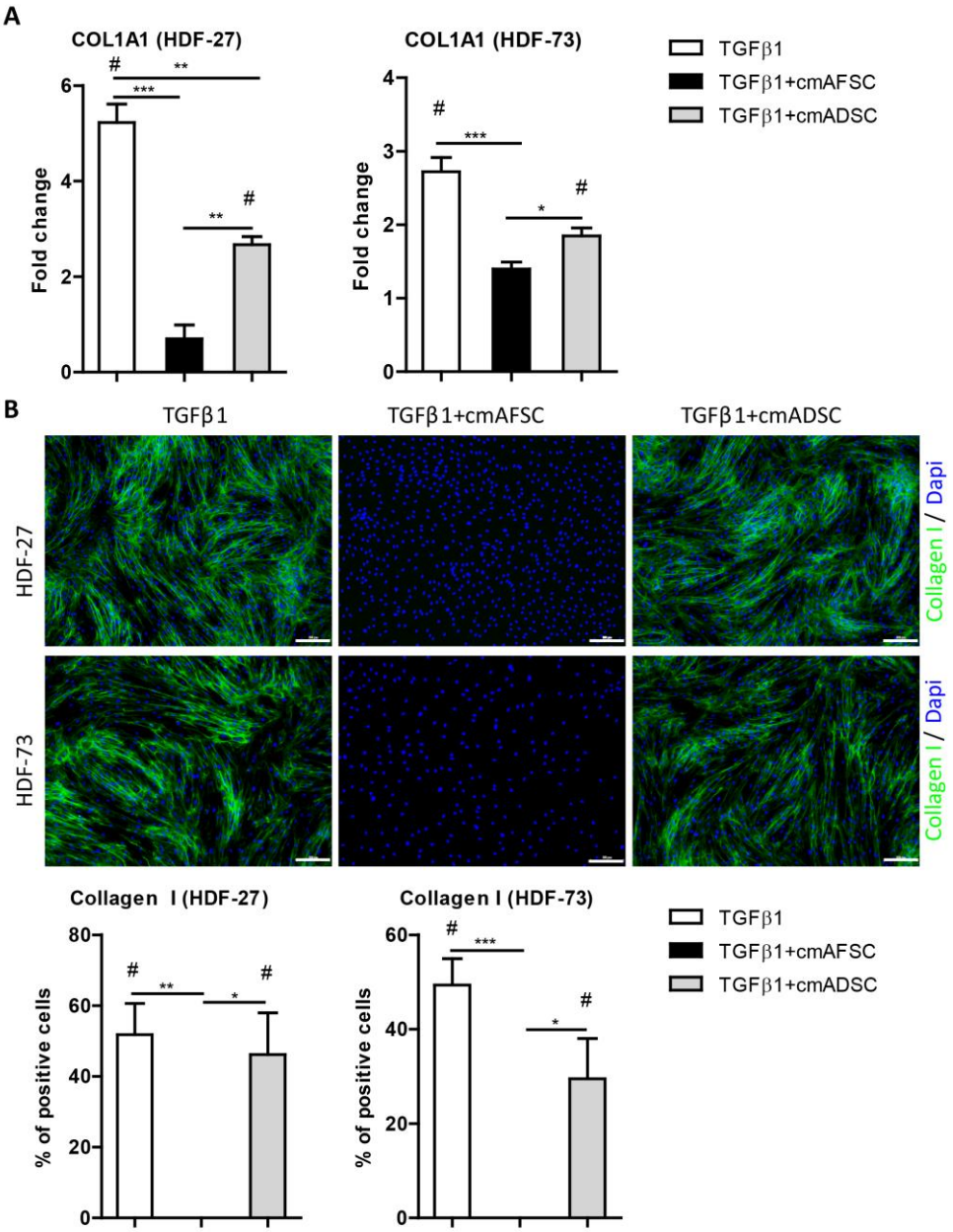


Figure 3. Effects of cmAFSCs and cmADSCs on TGFβ1-induced production of collagen type I in HDF-27 and HDF-73. Fibroblasts were treated with or without TGFβ1 and in combination with either CM of AFSCs or ADSCs for 48 h. (A) mRNA levels of COL1A1 as measured with qRT-PCR and expressed as fold-change compared to untreated control (cells cultured in plain medium without TGFβ1). (B) Representative immunofluorescence stainings and quantification of the % of cells positive for collagen type I in HDF-27 (upper panel) and HDF-73 (lower panel). White scale bar represents 200 µm. The sign # represents statistically significance towards the untreated control.

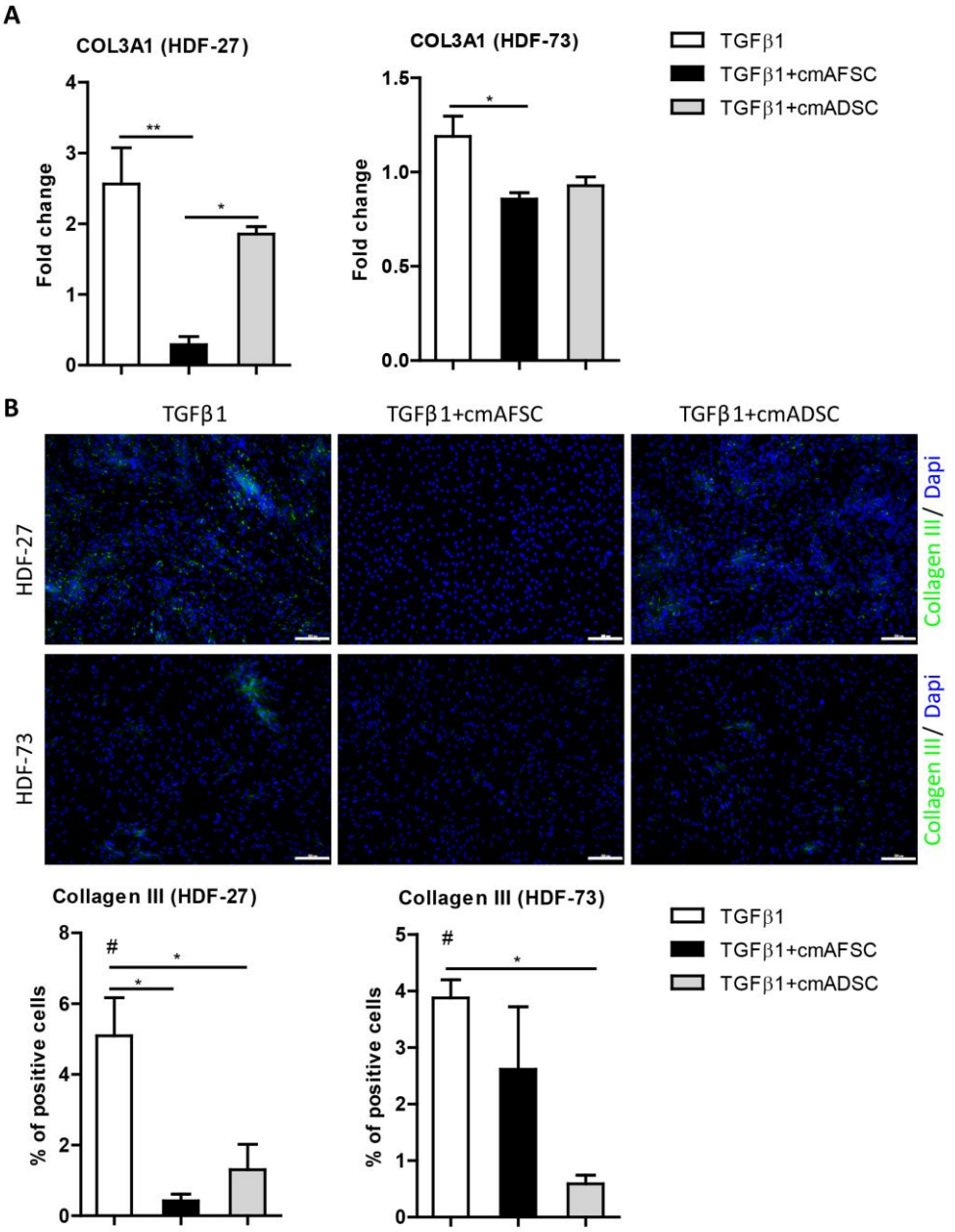


Figure 4. Effects of cmAFSCs and cmADSCs on TGFβ1-induced production of collagen type III in HDF-27 and HDF-73. Fibroblasts were treated with or without TGFβ1 and in combination with either CM of AFSCs or ADSCs for 48 h. (A) mRNA levels of COL3A1 are measured with qRT-PCR and expressed as fold-change compared to untreated control (cells cultured in plain medium without TGFβ1). (B) Representative immunofluorescence stainings and quantification of the % of cells positive for collagen type III in HDF-27 (upper panel) and HDF-73 (lower panel). White scale bar represents 200 µm. The sign # represents statistically significance towards the untreated control.

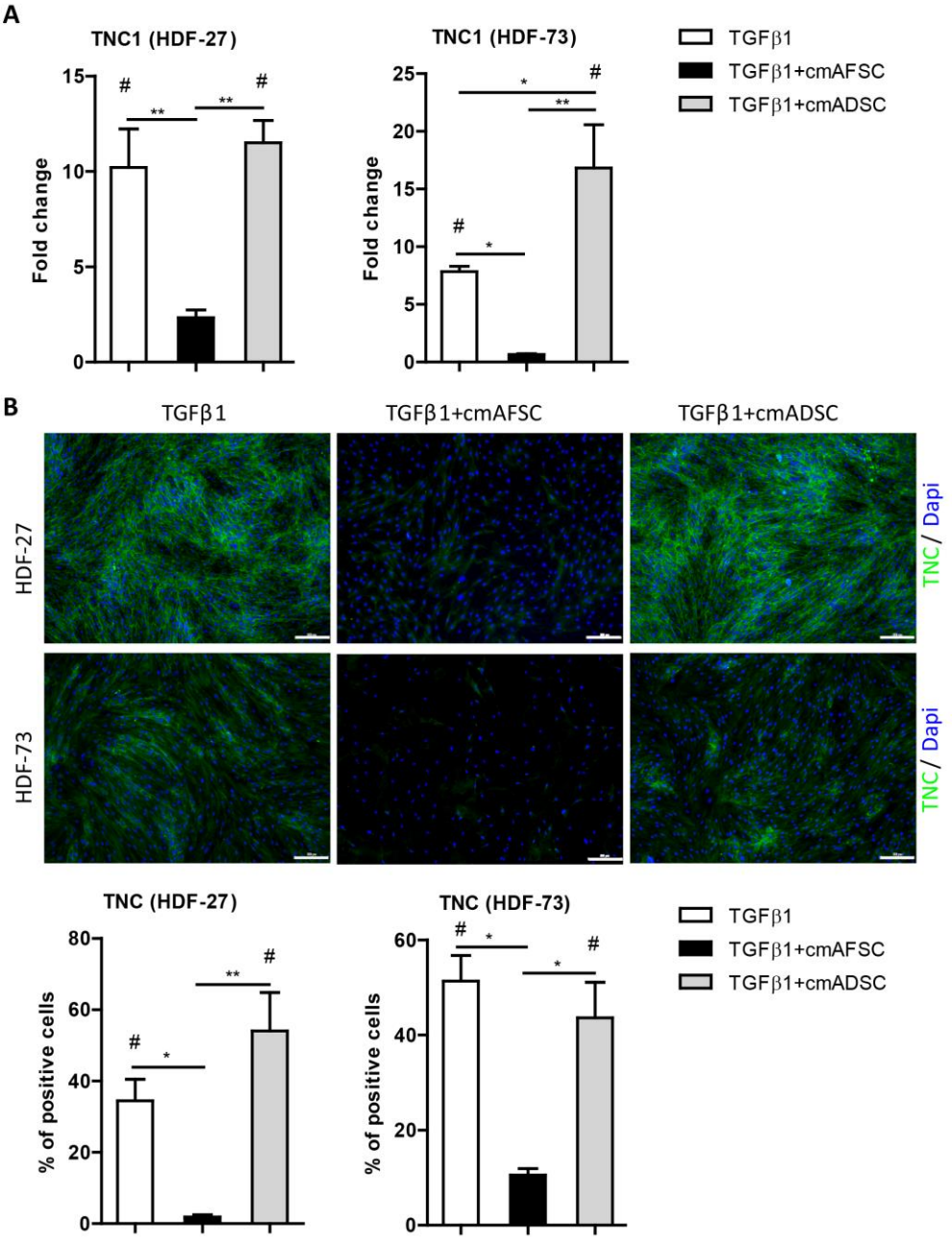


Figure 5. Effects of cmAFSCs and cmADSCs on TGFβ1-induced synthesis of tenascin C in HDF-27 and HDF-73. Fibroblasts were treated with or without TGFβ1 and in combination with either CM of AFSCs or ADSCs for 48 h. (A) mRNA levels of TNC1 as measured with qRT-PCR and expressed as fold-change compared to untreated control (cells cultured in plain medium without TGFβ1). (B) Representative immunofluorescence stainings and quantification of the % of cells positive for TNC in HDF-27 (upper panel) and HDF-73 (lower panel). White scale bar represents 200 µm. The sign # represents statistically significance towards the untreated control.

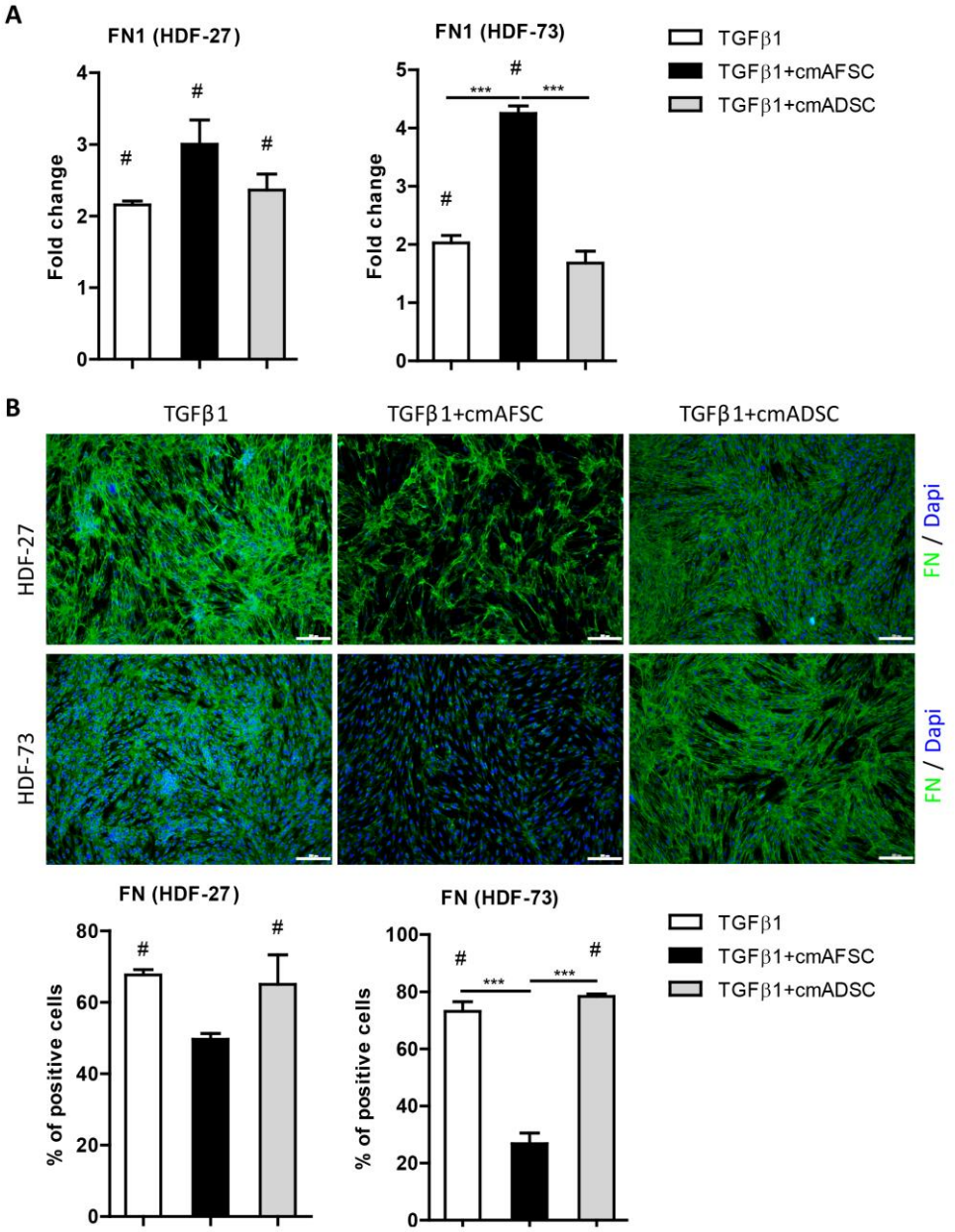


Figure 6. Effects of cmAFSCs and cmADSCs on TGFβ1-induced synthesis of fibronectin in HDF-27 and HDF-73. Fibroblasts were treated with or without TGFβ1 and in combination with either CM of AFSCs or ADSCs for 48 h. (A) mRNA levels of FN1 were measured with qRT-PCR and expressed as fold-change compared to untreated control. (B) Representative immunofluorescence stainings and quantification of the % of cells positive for FN in HDF-27 (upper panel) and HDF-73 (lower panel). White scale bar represents 200 µm. The sign # represents statistically significance towards the untreated control.

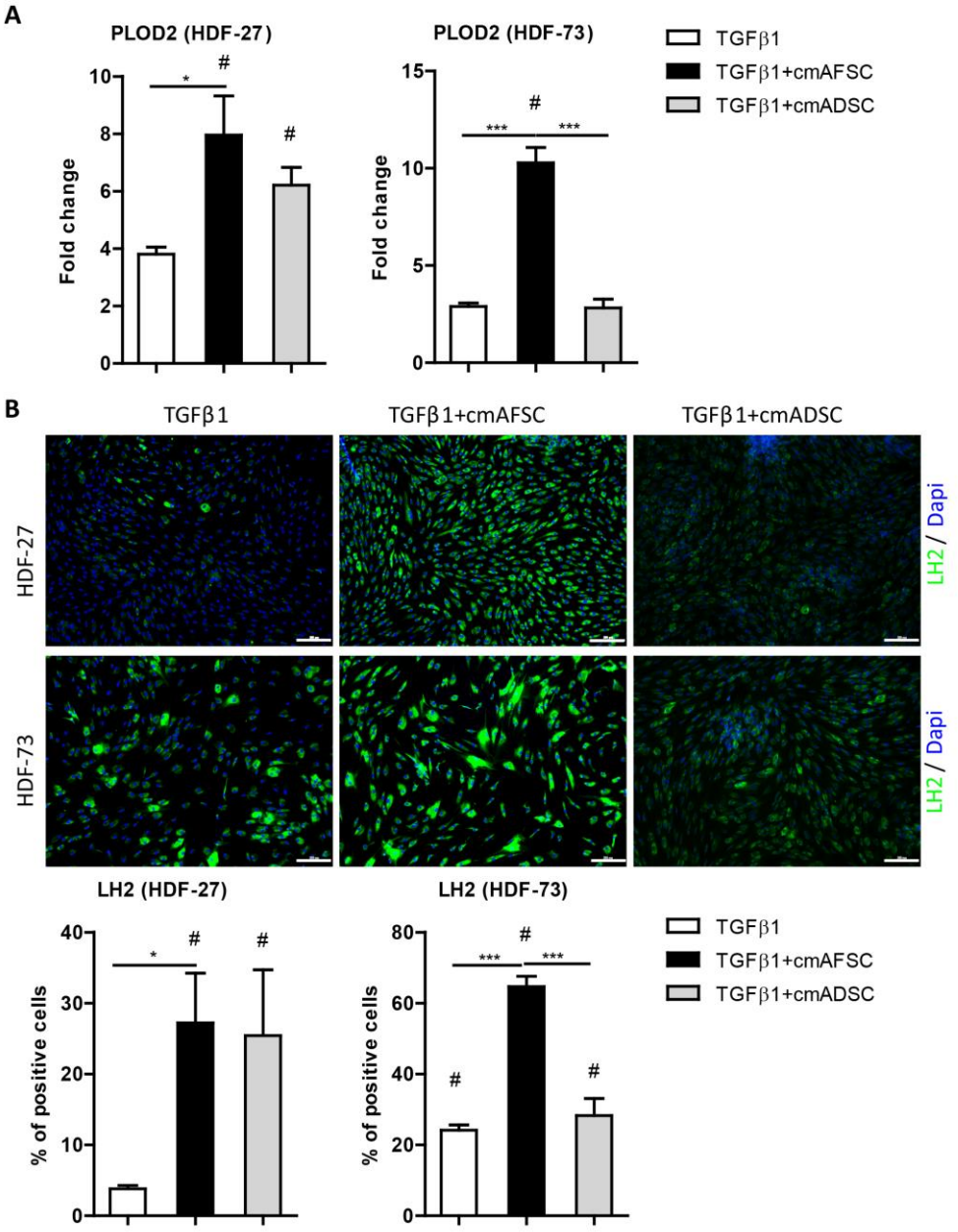
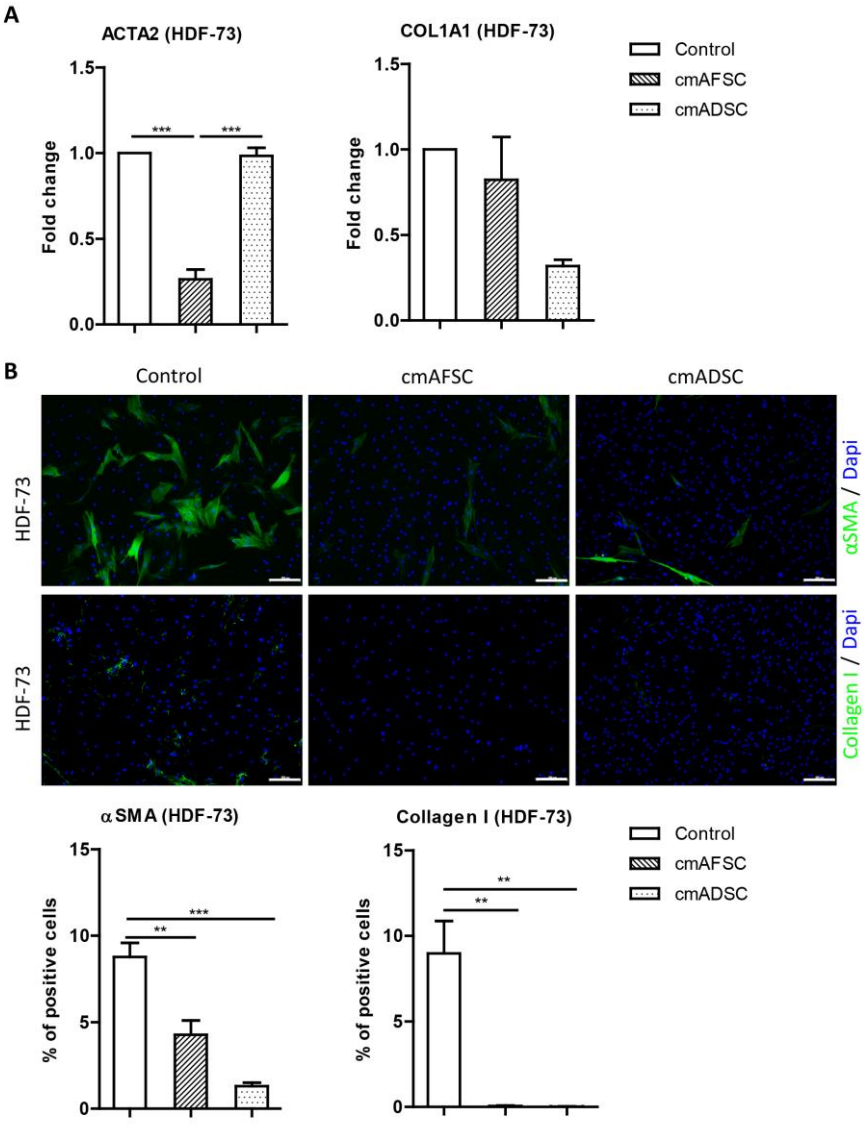


Figure 7. Effects of cmAFSCs and cmADSCs on TGFβ1-induced production of the collagen-modifying enzyme lysyl hydroxylase 2 (PLOD2) in HDF-27 and HDF-73. Fibroblasts were treated with or without TGFβ1 and in combination with either CM of AFSCs or ADSCs for 48 h. (A) mRNA levels of PLOD2 were measured with qRT-PCR and expressed as fold-change compared to untreated control. (B) Representative immunofluorescence stainings and quantification of the % of cells positive for PLOD2 in HDF-27 (upper panel) and HDF-73 (lower panel). White scale bar represents 200 µm. The sign # represents statistically significance towards the untreated control.

Effect of cmAFSCs and cmADSCs on myofibroblast reversal and collagen synthesis

Since CM of AFSCs has such a major impact on myofibroblast formation and collagen synthesis, we wondered whether cells that are already myofibroblasts can be reversed by cmAFSCs. Since the fibroblast pool derived from the 73-year old donor (HDF-73) already showed myofibroblasts under normal culture conditions (i.e. without the addition of TGFβ1), as well as collagen protein production (Figure 1B), we investigated the effect of cmAFSCs on those cells. We found a significant decrease of αSMA-positive cells and collagen type I synthesis, an effect that, interestingly, could also be achieved with cmADSCs (Figure 8A-B).



Effect of bFGF-neutralizing antibodies on the cmAFSCs induced suppression of αSMA and collagen type I in HDF-73

The suppression of TGFβ1-induced αSMA and collagen type I expression by cmAFSCs to baseline levels is remarkable. A growth factor reported to be present in cmAFSCs but absent in conditioned medium of adult mesenchymal stem cells is basic fibroblast growth factor (bFGF, also known as FGF-2) [44]. bFGF is able to suppress TGFβ1-induced αSMA and collagen type I expression [45-47]. We investigated whether bFGF-neutralizing antibodies were able to reverse the effect of cmAFSCs on αSMA and collagen type I expression by HDF-73. Indeed, mRNA levels of ACTA2 and COL1A1 were reversed to the level as seen with TGFβ1 stimulation (Figure 9A). However, αSMA protein staining revealed only a partial reversal, and collagen type I protein staining showed no reversal at all (Figure 9B), even when the amount of neutralizing antibodies was increased to 5.0 μg/ml (data not shown). Thus, the presence of bFGF in cmAFSCs only seems to be partially responsible for the observed suppression of αSMA cytoskeletal formation by cmAFSCs, and does not seem to be involved in the suppression of collagen type I protein synthesis.

Figure 8. Effects of cmAFSCs and cmADSCs on αSMA and collagen type I synthesis in HDF-73. Fibroblasts were treated with or without CM of AFSCs or ADSCs for 48 h. (A) mRNA levels of ACTA2 and COL1A1 were measured with qRT-PCR and expressed as fold change compared to untreated control. (B) Representative immunofluorescence stainings and quantification of the % of cells positive for αSMA and collagen type I in HDF-73 are shown. White scale bar represents 200 μm.

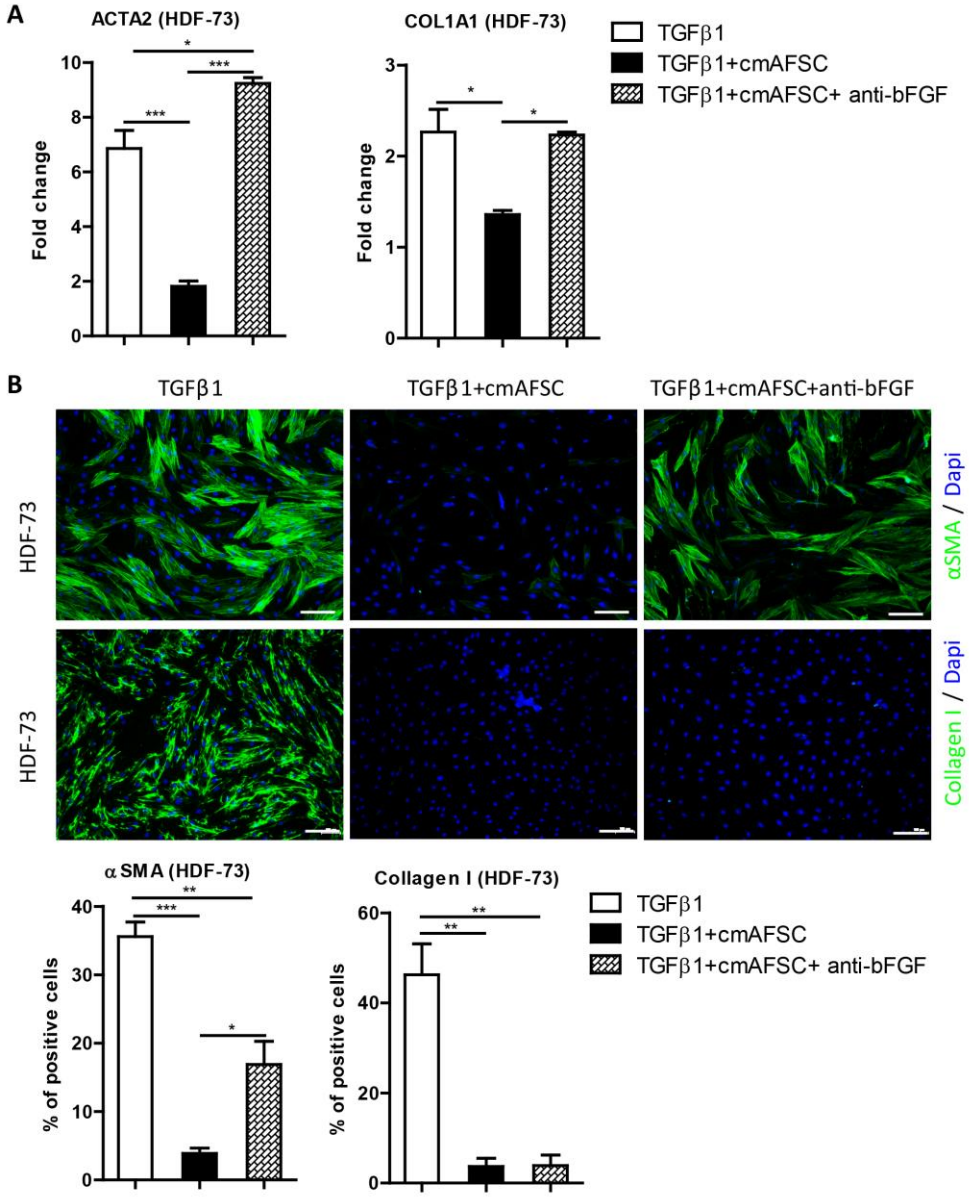


Figure 9. Effects of bFGF-neutralizing antibodies on αSMA and collagen synthesis in TGFβ1-stimulated HDF-73. Fibroblasts were treated with or without cmAFSCs in the presence or absence of bFGF-neutralizing antibodies for 48 h. (A) mRNA levels of ACTA2 and COL1A1 were measured with qRT-PCR and expressed as fold-change. (B) Representative immunofluorescence stainings and quantification of the % of cells positive for αSMA and collagen type I. White scale bar represents 200 µm.

Effect of cmAFSCs and cmADSCs on lysyl hydroxylase 2 synthesis in unstimulated fibroblasts

Since CM of AFSCs has an additive effect on PLOD2 expression (mRNA and protein level) in cells stimulated with TGFβ1 (both HDF-27 and HDF-73), whereas CM of ADSCs did not show such an effect, we investigated whether the same is the case in cells that are not exposed to TGFβ1. Indeed, the same effect was seen on the protein levels of lysyl hydroxylase 2, indicating that cmAFSCs is capable of highly up-regulating PLOD2 synthesis in both stimulated and unstimulated cells, a capacity that seems to be missing in cmADSCs (Figure 10).

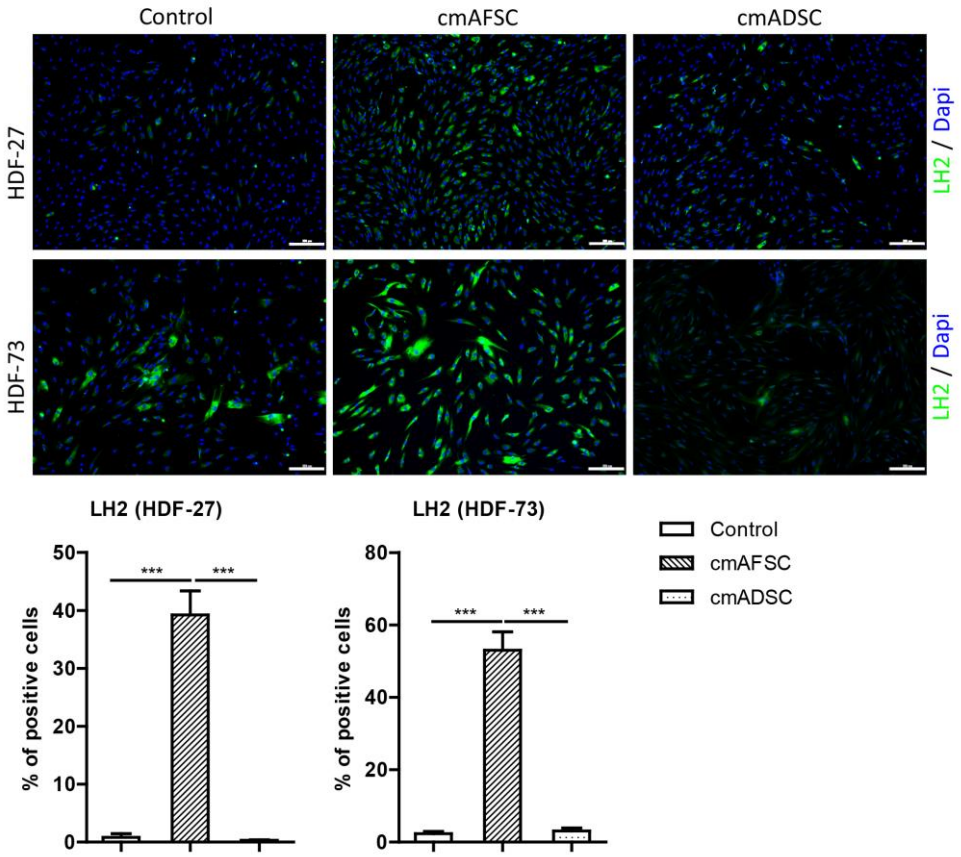


Figure 10. Effects of cmAFSCs and cmADSCs on the production of intracellular collagen-modifying enzyme PLOD2 in HDF-27 and HDF-73. Fibroblasts were treated with or without CM of AFSCs or ADSCs for 48 h. Representative immunofluorescence stainings and quantification of the % of cells positive for PLOD2 in HDF-27 (upper panel) and HDF-73 (lower panel) are shown. White scale bar represents 200 µm.

Effect of cmAFSCs and cmADSCs on (myo)fibroblast proliferation

We wondered whether the proliferation of (myo)fibroblasts change in response to CM. The proliferation marker Ki-67 revealed that there was a significant increase of proliferation of HDF-27 and HDF-73 when treated with cmAFSCs. However, this effect was not observed with cmADSCs. Similar results were obtained in a TGFβ1-rich environment (Figure 11).

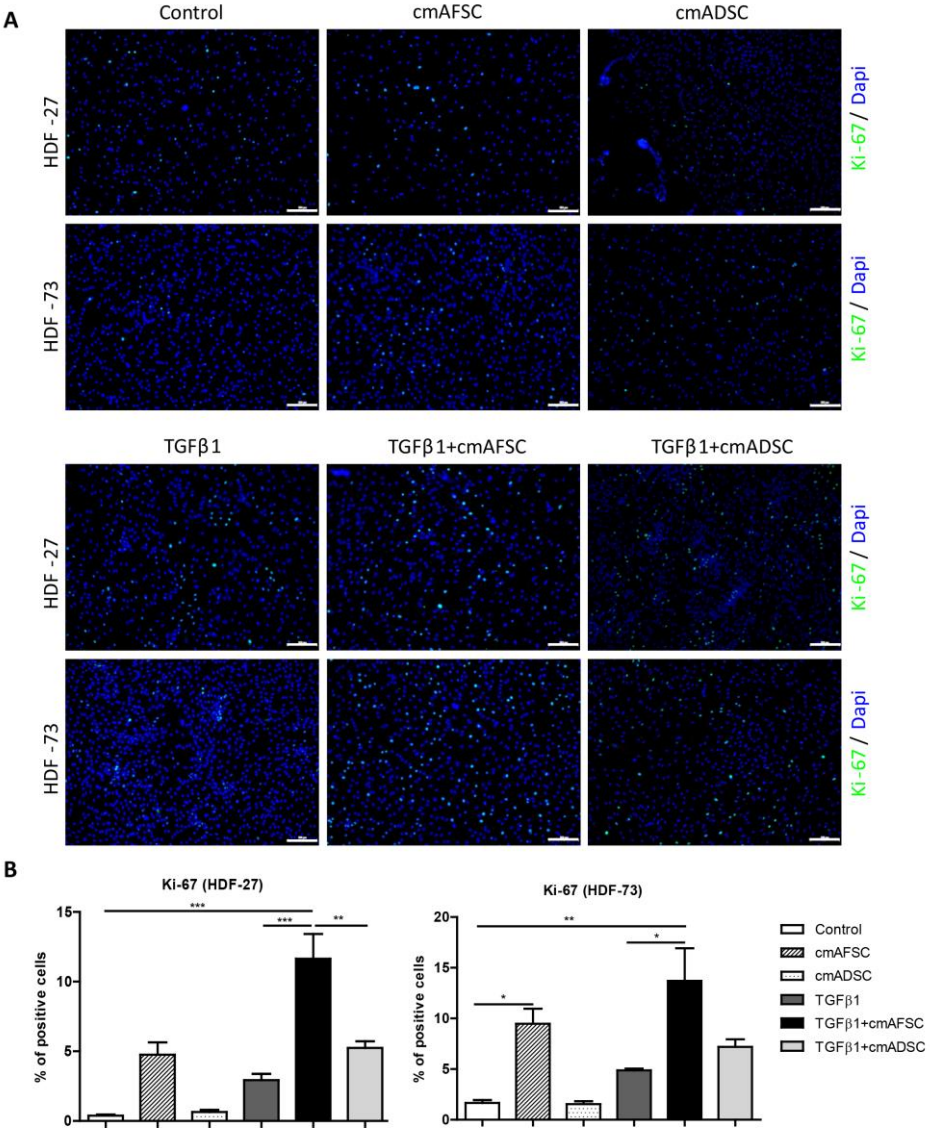


Figure 11. Effects of cmAFSCs and cmADSCs on the proliferation (Ki-67) of HDF-27and HDF-73. Fibroblasts were treated with or without TGFβ1 and in combination with either CM of AFSCs or ADSCs for 48 h. (A) Representative immunofluorescence stainings and (B) quantification of the % of cells positive for Ki-67 in HDF-27 and HDF-73. White scale bar represents 200 µm.

Discussion

Myofibroblasts play a central role in fibrosis, as it is the myofibroblast that is responsible for the pathological accumulation of collagen (i.e., the hallmark of fibrosis). Myofibroblasts can be obtained by stimulating fibroblasts with TGFβ. Although we observed an increase in collagen type I mRNA levels after TGFβ stimulation (3 to 5-fold), a much more dramatic increase is observed in collagen protein levels. That collagen protein synthesis is associated with myofibroblasts and not with fibroblasts could also be observed in the two non-stimulated fibroblast pools. The HDF-73 donor already showed substantial myofibroblast numbers whereas the HDF-27 donor showed only sporadically myofibroblasts, and this was reflected in collagen synthesis levels (and again: not in mRNA levels, as there was no significant difference in COL1A1 mRNA levels between HDF-73 and HDF-27). Because of this major discrepancy between collagen mRNA and protein levels between fibroblasts and myofibroblasts, care should be taken in drawing conclusions about anti-fibrotic properties if only mRNA levels are taken into consideration. Even more importantly, the data show that in fibroblasts a mechanism is operational that inhibits collagen synthesis at a transcriptional level, but that in myofibroblasts this mechanism is not operational anymore. It seems that this has, so far, not been realized in literature.

In preclinical models it has been shown that CM from stem cells attenuates fibrosis, but limited data are available on the effect of CM on (myo)fibroblasts *in vitro*. Here we show that cmAFSCs completely abrogates the TGFβ-induced activation of fibroblasts into myofibroblasts and, indeed, the formation of collagen type I molecules. In addition, pre-existing myofibroblasts were reversed into fibroblasts, with a concomitant attenuation of collagen synthesis. Thus, CM of amniotic fluid-derived stem cells seems to have strong anti-fibrotic properties as it can directly act on (myo)fibroblasts. The data on the suppression of collagen type III and tenascin C mRNA/protein levels in TGFβ1-stimulated HDF-27 by cmAFSCs support this. The effect was seen irrespective of differences of passage number between HDF-27 and HDF-73, age of the donor, or anatomical location of the fibroblasts (forearm versus face), showing the robustness of the anti-fibrotic properties of cmAFSCs.

Proteomic analysis of conditioned medium of AFSCs and adult mesenchymal stem cells has shown that cmAFSCs contains several growth factors in a considerable higher concentration [44]. Such a growth factor is bFGF; interestingly, it has been reported that bFGF is able to downregulate αSMA and collagen protein synthesis [45-47]. Indeed, neutralizing antibodies against bFGF partially neutralized the cmAFSCs-induced suppression of cytoskeletal αSMA formation, showing that the bFGF in cmAFSCs is at least partially involved in inhibiting myofibroblast formation. No effect was seen in collagen synthesis, indicating that in cmAFSCs a factor other than bFGF is involved in suppressing collagen protein synthesis.

cmADSCs showed markedly less anti-fibrotic properties in the context of TGFβ-induced activation of fibroblasts into myofibroblasts: suppression (if at all) of mRNA levels of ACTA2 did not reached baseline levels, as was the case with αSMA staining. The same was observed with collagen type I mRNA and protein levels. In contrast, pre-existing myofibroblasts were reversed into fibroblasts, with a concomitant decrease of collagen synthesis. Thus, CM of adipose tissue-derived stem cells seems to have anti-fibrotic properties, but under more limited conditions only.

So far, all published papers dealing with human fibroblasts report an up-regulation of collagen after exposure of CM derived from fetal or adult stem cells [23, 24, 28-34, 37, 38], which is in sharp contrast to our observations. The explanation for this discrepancy is simple. First, none of the published studies challenged fibroblasts with TGFβ, thus investigating the reaction of normal fibroblasts (not myofibroblasts) that show baseline levels of collagen only. Second, and more importantly, none of the studies added L-ascorbic acid (or better: its stable derivative L-ascorbic acid 2-phosphate) in the culture medium. It is known for a long time that L-ascorbic acid, better known as vitamin C, is required for collagen protein synthesis [48-50], for example by being the co-factor for prolyl hydroxylase, an enzyme that converts proline residues of the α-chains of collagen into hydroxyproline [51, 52]. This amino acid stabilizes the triple helix; a decreased prolyl hydroxyation of the α-chains results in unstable collagen molecules [52, 53]. Such molecules are degraded intracellularly and are not transported out of the cell. Human (myo)fibroblasts cultured without vitamin C therefore produce relatively small amounts of collagen, if any. In contrast to human fibroblasts, all published papers dealing with rodent (mice, rats) fibroblasts report a down-regulation of collagen synthesis after the addition of CM [5, 6, 25, 26, 35, 36]. Although in these studies vitamin C is also not added, it is a well-known fact that rodent fibroblasts synthesize their own vitamin C, while human cells are not able to do so (due to the absence of the enzyme L-gulonolactone oxidase) [54]. Human fibroblasts rely *in vivo* on the vitamin C levels present in the micro-environment in which they are embedded.

Our data on collagen obtained with CM-treated human dermal fibroblasts cultured in the presence of vitamin C are in line with those published with rodent fibroblasts. We also show a down-regulation of αSMA, which has been reported for rodent fibroblasts as well [5, 6, 25, 26], even in the presence of TGFβ1 [25, 26]. Interestingly, the anti-fibrotic property of CM is not species-specific, and not even cell-specific. CM of human amniotic epithelial cells suppresses collagen and αSMA (both on a protein level) in both rodent cardiac fibroblasts and rodent stellate cells [25, 26]. CM of rodent adult mesenchymal stem cells suppresses collagen and αSMA in rodent cardiac fibroblasts [25, 26, 35, 36]. Our paper is the first that describe the anti-fibrotic properties of CM of human mesenchymal stem cells (both amniotic fluid-derived and adipose tissue-derived) towards human fibroblasts (dermal, both adult and of older adult age).

Lysyl hydroxylase 2 (encoded by PLOD2) has been shown to be involved in fibrotic conditions by hydroxylating lysyl residues in collagen telopeptides [55, 56], resulting in collagen molecules that are cross-linked via pyridinolines. Such collagen molecules are difficult to degrade by collagenases, thus contributing to the accumulation of collagen [57]. Much to our surprise, cmAFSCs was able to highly upregulate lysyl hydroxylase 2 protein synthesis, both in unstimulated and in TGFβ1-stimulated fibroblasts. Since we could not detect collagen synthesis in cells treated with cmAFSCs, we do not have an obvious explanation for this up-regulation, but it is possible that lysyl hydroxylase 2 has a function other than hydroxylating lysyl residues in collagen telopeptides.

It is well-known that fetal skin wounds heal without scarring while healing of adult tissue leads to scar formation [39, 40, 58]. Although it has been suggested that scarless fetal skin healing properties are intrinsic to fetal skin and are not primarily the result of the fetal environment [59], we here show that the paracrine factors of amniotic fluid stem cells create a more powerful anti-fibrotic environment than adult stem cells. From these observations it appears that especially cmAFSCs has potential wound healing properties for adult skin, and most likely also for other organs that are prone to scarring.

Conclusions

In conclusion, we have demonstrated in this study the robust beneficial anti-fibrotic effects of conditioned medium of AFSCs. The secreted products of AFSCs might be employed in anti-fibrotic strategies, e.g. in regenerative medicine to enhance functional tissue repair. AFSCs can be obtained noninvasively, are highly abundant, and there are no ethical barriers to use these cells in clinical applications. To date, there are no effective drugs to treat fibrotic diseases. Our study might pave the way for the clinical application of conditioned medium in anti-fibrotic therapies; after all, therapies with paracrine factors are more practical than cell therapies.

Abbreviations

ACTA2: actin, alpha 2; AFSCs: amniotic fluid-derived stem cells; ADSCs: adipose tissue-derived stem cells; bFGF: basic fibroblast growth factor; BSA: bovine serum albumin; COL1A1: collagen type I, alpha 1; COL3A1: collagen type III, alpha 1; CM: conditioned medium; DMEM: Dulbecco's Modified Eagle Medium; EMEM: Eagle's Minimal Essential Medium; ECM: extracellular matrix; FN1: Fibronectin 1; HDF: human dermal fibroblasts; LH2: lysyl hydroxylase 2; PLOD2: procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2; qRT-PCR: quantitative real time polymerase chain reaction; TGFβ1: transforming growth factor beta 1; TNC1: tenascin C; αSMA: alpha smooth muscle actin; YWHAZ: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide

Competing interests

The authors declare that they have no competing interests.

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